• COLORECTAL CANCER •

Methylation profile of the promoter CpG islands of 31 genes that may contribute to colorectal carcinogenesis

Xiao-Li Xu, Jian Yu, Hong-Yu Zhang, Meng-Hong Sun, Jun Gu, Xiang Du, Da-Ren Shi, Peng Wang, Zhen-Hua Yang, Jing-De Zhu

Xiao-Li Xu, Meng-Hong Sun, Xiang Du, Da-Ren Shi, Department of Pathology, Cancer Hospital, Fudan University, Shanghai 200032, China
Jian Yu, Hong-Yu Zhang, Jun Gu, Peng Wang, Jing-De Zhu, The State-Key Laboratory for Oncogenes and Related Genes, Cancer Institute of Shanghai Jiaotong University, Shanghai 200032, China
Zhen-Hua Yang, Institute of Respiratory Disease, Zhong Shan Hospital, Fudan University, Shanghai 200032, China
Co-first-authors: Xiao-Li Xu, Jian Yu, Hong-Yu Zhang
Correspondence to: Jing-De Zhu, The State-Key Laboratory for Oncogenes and Related Genes, Cancer Institute of Shanghai Jiaotong University, LN2200/25, Xietu Road, Shanghai 200032, China. zhujingde@yahoo.com
Telephone: +86-21-64224285   **Fax:** +86-21-64224285

Abstract

AIM: To establish the methylation profile of the promoter CpG islands of 31 genes that might play etiological roles in colon carcinogenesis.

METHODS: The methylation specific PCR in conjunction of sequencing verification was used to establish the methylation-profile of the promoter CpG islands of 31 genes in colorectal cancer ($n = 65$), the neighboring non-cancerous tissues ($n = 5$), colorectal adenoma ($n = 8$), and normal mucosa ($n = 1$). Immunohistochemically, expression of 10 genes was assessed on the home-made tissue microarrays of tissues from 58 patients. The correlation of tumor specific changes with each of clinical-pathologic features was scrutinized with relevant statistic tools.

RESULTS: In comparison with the normal mucosa of the non-cancer patients, the following 14 genes displayed no tumor associated changes: breast cancer 1, early onset (*BRCA1*), cadherin 1, type 1, E-cadherin (epithelial) (*CDH1*), death-associated protein kinase 1 (*DAPK1*), DNA (cytosine-5-)-methyltransferase 1 (*DNMT1*), melanoma antigen, family A, 1 (directs expression of antigen MZ2-E) (*MAGEA1*), tumor suppressor candidate 3 (*N33*), cyclin-dependent kinase inhibitor 1A (p21, Cip1) (*p21^{WAF1}*), cyclin-dependent kinase inhibitor 1B (p27, Kip1) (*p27^{KIP1}*), phosphatase and tensin homolog (mutated in multiple advanced cancers 1) (*PTEN*), retinoic acid receptor, beta (*RAR-*, Ras association (RalGDS/AF-6) domain family 1 C (*RASSF1C*), secreted frizzled-related protein 1 (*SFRP1*), tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory) (*TIMP3*), and von Hippel-Lindau syndrome (*VHL*). The rest 17 targets exhibited to various extents the tumor associated changes. As changes in methylation of the following genes occurred marginally, their impact on the formation of colorectal cancer were trivial: adenomatous polyposis coli (*APC*) (8%, 5/65),

Ras association (RalGDS/AF-6) domain family 1A (*RASSF1A*) (3%, 2/65) and cyclin-dependent kinase inhibitor 2A, alternated reading frame (*p14^{ARF}*) (6%, 4/65). The following genes exhibited moderate changes in methylation: O-6-methylguanine-DNA methyltransferase (*MGMT*) (20%, 13/65), mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli) (*hMLH1*) (18%, 12/65), cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4) (*p16^{INK4a}*) (10%, 10/65), methylated in tumor 1 (*MINT1*) (15%, 10/65), methylated in tumor 31 (*MINT31*) (11%, 7/65). The rest changed greatly in the methylation pattern in colorectal cancer (CRC): cyclin A1 (*cyclin a*1) (100%, 65/65), caudal type homeobox transcription factor 1 (*CDX1*) (100%, 65/65), *RAR-* (85%, 55/65), myogenic factor 3 (*MYOD1*) (69%, 45/65), cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4) (*p15^{INK4b}*) (68%, 44/65), prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (*COX2*) (72%, 47/65), cadherin 13, H-cadherin (heart) (*CDH13*) (65%, 42/65), CAAX box 1 (*CXX1*) (58%, 38/65), tumor protein p73 (*p73*) (63%, 41/65) and Wilms tumor 1 (*WT1*) (58%, 38/65). However, no significant correlation of changes in methylation with any given clinical-pathological features was detected. Furthermore, the frequent changes in methylation appeared to be an early phase event of colon carcinogenesis. The *in situ* expression of 10 genes was assessed by the immunohistochemical approach at the protein level: *CDH1, CDH13, COX2, cyclin A1, hMLH1, MGMT, p14^{ARF}, p73, RAR-*, and *TIMP3* genes in the context of the methylation status in colorectal cancer. No clear correlation between the hypermethylation of the promoter CpG islands and the negative expression of the genes was established.

CONCLUSION: The methylation profile of 31 genes was established in patients with colon cancer and colorectal adenomas, which provides new insights into the DNA methylation mediated mechanisms underlying the carcinogenesis of colorectal cancer and may be of prognostic values for colorectal cancer.

INTRODUCTION

Colorectal carcinoma (CRC) is the third most common malignancy in the developed countries, with an annual incidence approximately 35-50/100 000[1,2]. The risk factors of CRC include Western diet (low fiber, high fat diet), family history, smoking, obesity and inflammatory colorectal diseases. As a whole, China falls into the low incidence country in the world, with the relative incidence of CRC rates being the 4th-6th of the total malignances and the 2nd-3rd of those in the digestive system in China. However, CRC

occurrence exhibits an increasing trend, with an average 4.2% increase per year in the last two decades[3]. CRC is the 3rd common malignancy in Shanghai and occurs more frequently in China (http://www.caca.org.cn/zlyy/shanghai/index.asp). Clinically and pathologically, CRC has been grouped as the sporadic (approximately 80% cases) and hereditary types of diseases (roughly 20% in the total).

Both activation of proto-oncogenes (e.g. the *CDH13 gene*) and inactivation of tumor suppressor genes (e.g. the *APC*, *p53* and genes responsible for the mismatch repaired activity in cells) attributed to genetic defects have been proved instrumental to carcinogenesis of colorectal cancer. The well-known adenoma-carcinoma sequence of the genetic defects by Volgestein has gained both experimental supports and general recognition. However, the predicted accumulation of genetic events has not been substantiated in sporadic colorectal adenocarcinomas, and the expected increase in the number of genetic events in aging tissues has not materialized[4]. Both observations are indeed at odds with the exponential increase in the incidence of colorectal cancer with age. Therefore, alternative mechanisms must be accounted for such a shortfall, which is associated with DNA methylation mediated control of the expression of critical genes overseeing cell growth, apoptosis, as well as cell cycle progression.

Addition of the methyl group at the fifth carbon of cytosine of CpGs is the only covalent DNA modification in vertebrate[5]. Such epigenetic signatures in parental cells are passed to the daughter cells at high fidelity, by a similar semi-conserved mechanism during duplication of the genetic information. The hypermethylated status of CpGs can affect the DNA-protein interaction by eliminating the otherwise occurring DNA-protein interactions involved with the methylation-sensitive transcription factors, while unfolding a cascade of reactions toward the condensed chromatin that is initiated with binding to the methylated CpG by members of the methylated CpG binding protein family[6]. Cancer specific changes in methylation occur widely in all the cancers tested[7]. The global demethylation of DNA has been linked to the increased genome instability as the otherwise transcription-/ transposition-silenced repetitive sequences in normal cells are reactivated by DNA demethylation[8,9]. Paradoxically, local hypermethylation (at the promoter CpG island) has been well established for inactivation of tumor suppressor genes and caught great attention. However, the local demethylation in human tumors has also been recently reported to be associated with transcription reactivation of the silenced genes in normal tissues, such as the *MAGEA1* gene in hepatocellular carcinoma[10]. In view of the fact that the majority of the promoter CpG island containing genes (50% of the protein-coding genes fall into this category) have not been properly looked in the context of the tumor biology, methylation profiling remains valuable for new insights into the etiological mechanism.

CRC is one of the first few types of cancers for methylation profiling[11,12]. Learnt from the rather extensive survey with more than 30 targets, a so called "CpG island methylation (CIM)" phenomenon has been reported, i.e., a clustering occurrence of multiple hypermethylated targets including upto a dozen of tumor suppressor genes[11]. In this study, we recruited 65 CRC tissues, eight colorectal adenomatous tissues, five neighboring non-cancerous tissues and one normal mucosa tissue from Shanghai Cancer Hospital for a middle scale methylation profiling with the promoter CpG islands of 31 genes, aiming, to establish the methylation profile of CRC patients in China, and to assess the association between the methylation status and expression profile of the genes *in situ*. Our results presented in this report should provide some new insights into the role of the epigenetic mechanism in CRC carcinogenesis.

MATERIALS AND METHODS
Clinical-pathological profiles of CRC patients
With the informed consent of all patients and approval of the ethics committee, tumor tissues were obtained during surgery from 65 CRC patients at the Cancer Hospital of Fudan University in 1996-2003. They were 39 patients with sporadic colorectal cancer (SCRC), 10 with hereditary non-polyposis colorectal cancer (HNPCC) fulfilling Amsterdam criteria of IGC and Japanese criteria, and 16 in the category of Bethesda CRC. In addition, tissues were taken from the neighboring non-cancerous mucosa (at least 5-10 cm away from the tumorous lesions) of five patients. A cecum sample was obtained from a 68-year old female patient with inflammatory pseudotumor. In addition, Bethesda CRC concomitant adenomatous samples from six patients and sporadic colon adenomatous samples (one was cancer concomitant) from two patients were taken.

All samples were freshly obtained and cut into two parts. One was buried into an optimal cutting temperature compound (OCT, Miles Inc, USA), and put into fluid nitrogen for storage. The rest was fixed in formalin, embedded in paraffin and then cut into 4-5 μm sections for routine pathological diagnosis.

Clinical-pathological features including the age and gender of patients, the tumor site histological type, Duke's stages, differentiation grades (only moderate and poor differentiation), lymph node metastasis status, and more than 3 years follow-up results (36 cases) are shown in Table 1. The clinical-pathologic data of the eight adenomas are shown in Table 2.

Table 1 Clinical-pathologic profiles of the CRC cases in this study

Clinical-pathologic features		Number of cases
Gender	Male	37
	Female	28
Age (yr)	Median	60
	Range	28-85
Tumor site	Left colon and rectum	43
	Right colon	22
Histological types	Adenocarcinoma	51
	Mucinousi carcinoma	5
	Other types combined[1]	9
Differentiation	Moderate	51
	Poor	14
Follow-up (3 yr)	Survival	27
	Deceased	9
Duke's stage	A	6
	B	15
	C	30
	D	14

[1]Other types combined included the mixed squamous cell carcinoma, carcinoid carcinoma, *etc.*

Table 2 Clinical-pathologic profiles of adenomas and neighboring tumor-free mucosae

Clinical-pathologic features		Adenoma	Mucosa
Gender	Male	4	2
	Female	4	4
Age (yr)	Median	60	68
	Range	34-83	47-76
Tumor site	Left colon	6	4
	Right colon	2	2
Histological type	Tubular	3	
	Tubulovillous	5	
Dysplasia	Light-moderate	8	

Table 3 Target CpG islands and the primers for MSP

Gene	GenBank No.	Primer Sense (5'-3')	Primer Anti-sense (5'-3')	Location to transcription start	Size (bp)
APC	U02509	(U) GTGTTTTATTGTGGAGTGTGGGTT	CCAATCAACAAACTCCCAACAA	-17 147 to -17 050	108
		(M) TATTGCGGAGTGCGGGTC	TCGACGAACTCCCGACGA	-17 153 to -17 046	97
BRCA1	L78833	(U) GGTTAATTTAGATTTTGAGAGATG	TCAACAAACTCACACCACACAATCA	-320 to -138	182
		(M) GGTTAATTTAGAGTTTCGAGAGACG	TCAACGAACTCACGCCGCGCAATCG	-320 to -138	182
CDH1	NM_004938	(U) GGTGGGTGGGTTGTTAGTTTTGT	AACTCACAAATCTTTACAATTCCAAC	-266 to -93	172
		(M) GTGGGCGGGTCGTTAGTTTC	CTCACAAATACTTTACAATTCCGACG	-265 to -93	172
CDH13	AB001090	(U) TTGTGGGGTTTGTTTTTTGT	AACTTTTCATTCATACACACA	-267 to -24	243
		(M) TCGCGGGGTTCGTTTTTCGC	GACGTTTTCATTCATACACGCG	-267 to -24	243
CDX1	NT_029289	(U) TGATTGGGTTGTTGTTTATGG	TACCACCACCACCTCCAA	+290 to +522	233
		(M) CGATTGGGTCGTCGTTTA	CCGCATCCACTCGTAAAA	+290 to +491	202
COX2	NT_004487	(U) TTGTTTGTTGTTGTGATGTTTG	TCCAAACTCTTTCCCAAATC	-119 to -324	206
		(M) GTTCGTCGTTGCGATGTT	CCAAACTCTTTCCCAAATCA	-121 to -323	203
CXX1	NT_011786	(U) TTAGGTTGGTTTTTGTGGATATG	CACCCAACCATCCATCAC	-24 to +92	117
		(M) AGGTCGGTTTTCGTGGAT	GATCGATATCGCCGTCAA	-22 to +192	215
Cyclina1	AF124143	(U) GGGTAGTTTTGTTGTGTTTTAGTTG	AACCACTAACAACCCCTCT	-762 to -565	199
		(M) TCGTCGCGTTTTAGTCGT	ACCCGTTCTCCCAACAAC	-755 to -550	206
DAPK1	NM_004938	(U) GGATAGTTGGATTGAGTTAATGTC	CAAATCCCTCCCAAACACCAA	-332 to -229	103
		(M) GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAAACGCCGA	-332 to -234	98
DNMT1	NT_011176	(U) GGGTGGTAGATGTTGTTTTTGT	CACCCTACCTATCCCCCTAA	-359 to -130	230
		(M) CGTCGTTTTCGTTATCGTT	ATACCTACCGCCTACGAACA	-320 to -140	181
hMLH1	AB017806	(U) TTTTGATGTAGATGTTTTATTAGGGTTGT	ACCACCTCATCATAACTACCCACA	-526 to -654	118
		(M) ACGTAGACGTTTTATTAGGGTCGC	CCTCATCGTAACTACCCGCG	-531 to -655	124
MAGEA1	U82670	(U) GTTTGGTTGAAGGAATTTGA	ACCCACAACCCTCCCTCTTA	+24 to +347	324
		(M) GTTCGGTCGAAGGAATTTGA	CCACAACCCTCCCTCTTAAA	+24 to +345	322
MGMT	AL355531	(U) TTTGTGTTTTGATGTTTGTAGGTTTTTGT	AACTCCACACTCTTCCAAAAACAAAACA	-451 to -266	209
		(M) TTTCGACGTTCGTAGGTTTTCGC	GCACTCTTCCGAAAACGAAACG	-469 to –261	186
MINT1	AF135501	(U) TATTTTTGAAGTGTTTGTTTGGTGT	TCCCTCTCCCCTCTAAACTTC		202
		(M) TTCGAAGCGTTTGTTTGG	CGCCTAACCTAACGCACA	*	160
MINT31	AF135531	(U) GGGTGGGAATTGAGATGATT	CATCACCACCCCTCACTTTA		131
		(M) GCGGGAATTGAGACGATT	ACGCTTACGCCACTACGA	*	176
MYOD1	NT_009307	(U) ATTTGATGGTTTTTGATGGTTT	CACACACATACTCATCCTCACA	+206 to +418	213
		(M) GACGGTTTTCGACGGTTT	GCCCGAAACCGAATACAC	+210 to +393	184
N33	NT_030737	(U) TTTGGTGAATTGGATGTTTTG	CACCCAACTCCTACCACACAC	+20 to +169	150
		(M) TTTTCGGTGAATCGGATG	TACGCGCCCAACTCCTA	+18 to +173	156
p14^{ARF}	L41934	(U) TGAGTTTGGTTTTGGAGGTGG	AACCACAACAACAAACACCCCT	+97 to +262	165
		(M) GTCGAGTTCGGTTTTGGAGG	AAAACCACAACGACGAACG	+95 to +255	160
p15^{INK4b}	NM_004936	(U) TGTGATGTGTTTGTATTTTGTGGTT	CCATACAATAACCAAACAACCAA	-318 to -164	154
		(M) GCGTTCGTATTTTGCGGTT	CGTACAATAACCGAACGACCGA	-312 to -165	147
p16^{INK4a}	NM_000077	(U) TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	-80 to +71	151
		(M) TTATTAGAGGGTGGGGCGGATCGC	ACCCCGAACCGCGACCGTAA	-80 to +69	149
p21^{WAF1}	NT_007592	(U) TTTTTGTAGTATGTGAGGTTTTGG	AACACAACTCAACACAACCCTA	-200 to -1	200
		(M) TGTAGTACGCGAGGTTTCG	TCAACTAACGCAACTCAACG	-196 to +5	202
p27^{KIP1}	AB003688	(U) TGTGATTTTGATGTTGGTAAGGT	CAAACCACAACCCAAACTCT	-363 to -141	223
		(M) CGACGTCGGTAAGGTTTG	AAACGCGCAAAAACTACG	-355 to -163	193
p73	AB031234	(U) TGGGTGTTTGGTTTGTAGGT	CCAACTCTCAACTCCCAAAA	-1 725 to -1 505	221
		(M) GCGTTCGGTTCGTAGGTT	CTCAACTCCCAAAACCCAA	-1 722 to -1 511	212
PTEN	NM_000314	(U) TGGTTTTTTGAGGTGTTTG	TTCCATCATAACTACAACTTCCA	-979 to -812	167
		(M) GGTTTTTCGAGGCGTTCG	CGCCTCACAACGACTCAACT	-978 to -786	192
RAR-β	NM_016152	(U) TTGAGAATGTGAGTGATTTGA	AACCAATCCAACCAAAACAA	-343 to -197	146
		(M) TCGAGAACGCGAGCGATTCG	GACCAATCCAACCGAAACGA	-343 to -197	146
RASSF1A	NT_022517	(U) TTTGGTTGGAGTGTGTTAATGTG	CAAACCCCACAAACTAAAAACAA	+70 to +178	108
		(M) GTGTTAACGCGTTGCGTATC	AACCCCGCGAACTAAAAACGA	+82 to +176	94
RASSF1C	NT_022517	(U) GGAGTTTGGATTGTTGGTTTTG	CACCCCCAAAAATAACCTCAT	-370 to -137	187
		(M) AGTTTGGATTGTCGGTTTCG	TCACAAACCCCACCTACCAC	-370 to -137	187
SFRP1	NT_008251	(U) GTTTTGTGGTTGTAAGTTGTTGTT	AAACCCCACACACTCCAA	-180 to +19	199
		(M) TCGCGGTCGTAAGTTGTT	CGCACTCCAACCCTACAA	-177 to +11	188
TIMP3	NM_000362	(U) TTTTGTTTTGTTATTTTTTGTTTTTGGTTTT	CCCCCAAAAACCCCACCTCA	-454 to -335	119
		(M) CGTTTCGTTATTTTTTGTTTTCGGTTTC	CCGAAAACCCCGCCTCG	-451 to -335	116
VHL	AF010238	(U) GTTGGAGGATTTTTTTGTGTATGT	CCCAAACCAAACACCACAAA	-185 to -20	165
		(M) TGGAGGATTTTTTTGCGTACGC	GAACCGAACGCCGCGAA	-183 to -25	158
WT1	X74840	(U) TGGGATTTGGGTGGTATTTG	CACCAACACCCACTACACCA	+295 to +510	216
		(M) GTTAGGCGTCGTCGAGGTTA	AAAACGCAAAATCCAACACC	+321 to +526	206

DNA preparation and methylation specific PCR analysis

Genomic DNA was extracted from frozen tissues by the protein precipitation method[13]. Frozen tissue block was cut into 10-20 μm sections, put into a 1.5 mL Eppendorf tube, and then incubated at 37 ℃ in the cellular lysis buffer with proteinase K (SABC, Shanghai) for a final concentration of 100 μg/mL. The digestion was carried out at 55 ℃ for at least 2 h. After centrifuged, the upper liquid was collected and the protein precipitation mix was added. After centrifuged, 1:1 isopropanol was added. The deposition was washed with 750 mL/L ethanol, and naturally dried. The DNA pellet was washed once with 700 mL/L ethanol and dissolved with TE (10 mmmol/L Tris HCl, pH 7.4 and 1 mmol/L EDTA), and stored at a concentration of 1 mg/mL at 4 ℃.

The primers for methylation specific PCR (MSP) (Table 3) were adopted as the previously published works[10,14] or designed with the assistance of the web server for the CpG islands identification (http://www.ebi.ac.uk/emboss/cpgplot/index.html) and the primer design software (http://micro-gen.ouhsc.edu/cgi-bin/primer3_www.cgi). DNA treatment and PCR reaction were carried out by the previously described method[10,14,15]. In detail, 10 μg DNA in 50 μL TE was incubated with 5.5 μL of 3 mol/L NaOH at 37 ℃ for 10 min, followed by a 16 h treatment at 50 ℃ after 30 μL of freshly prepared 10 mmol/L hydroquinone and 520 μL of freshly prepared 3.6 mol/L sodium-bisulfite at pH 5.0 were added. The DNA was desalted using a home dialysis system with 10 g/L agarose. The DNA in the desalted sample (approximately 100 μL in volume) was denatured at 37 ℃ for 15 min with 5.5 μL of 3 mol/L NaOH followed by ethanol precipitation with 33 μL 10 mol-L NH4OAc and 300 μL ethanol. After washed with 700 mL/L ethanol, the gently dried DNA pellet was dissolved with 30 μL TE at 65 ℃ for 10 min. DNA samples were finally stored at -20 ℃ until further use. The sample of 50 ng of DNA was reserved for PCR reaction. PCR reaction was carried out in a volume of 15 μL with 50 ng or less template DNA with FastStart Taq polymerase (Roche, Germany) as follows. After an initial denaturing step for 3 min at 94 ℃, 35 cycles at 94 ℃ for 30 s, at varying temperatures with primer pairs for 30 s and at 72 ℃ for 30 s, were carried out. The PCR products were separated by 12 g/L ethidium bromide containing agarose gel electrophoresis with 1×TAE and visualized under UV illumination. To verify the PCR results, representative bands from each target were gel-purified. Then the PCR products were sequenced directly or cloned into T-vector (Promega, USA) followed by automatic DNA sequencing provided by Bioasia (Shanghai, China). Only verified results were presented in this report.

Immunohistochemistry analysis on a homemade tissue microarray

The paraffin embedded tissues from 58 cancer cases were made into the tissue microarray paraffin blocks, followed by sectioning. For each sample, the H&E stained sections were first reviewed and marked for the picked point. At least 4 points of cancer tissue and 1 point of normal mucosa tissue were taken from the different positions on the paraffin block. The diameter of our tissue microarray punch was 1 mm. We put about 40-80 tissue dots in one array. To identify the tissue put into the array, all the made tissue microarray sections were stained with H&E and reviewed by two pathologists independently.

The immunostaining was performed with each of 10 commercially available antibodies (Table 4) using DAKO Envision system/3,3-diaminoben-aidine (DAB) staining. The result was scored by conjunction with both staining intensity and the percentage of positive staining cells. Each sample was given an intensity score (0-3) and a percentage of cell positive score (0 = less than 5%, 1 = 5-25%, 2 = 25-50%, 3 = 50-75%, 4 = more than 75%). An overall immunohistochemistry score was calculated by multiplying the intensity and percentage of cell

positive scores. Scores of 1-4 were recorded as +, 6-8 as ++, and 9-12 as +++.

Table 4 Antibodies for immunohistochemical analysis

Antibody name	Expression location	Company	Catalogue No.
CDH1	Membrane/Cytoplasm	Antibody Diagnostica INC.	M-0536
CDH13	Cytoplasm	Santa Cruz	sc-7940
COX2	Cytoplasm	Santa Cruz	sc-1746
Cyclin a1	Nuclear	Novocastra Lab.	NCL-CYCLINA
hMLH1	Nuclear	PharMingen	G168-15
MGMT	Cytoplasm	DAKO	M3610
p14[ARF]	Nulear/Cytoplasm	Santa Cruz	sc-8340
p73	Cytoplasm	Santa Cruz	sc-17823
RAR	Nuclear/ Cytoplasm	Santa Cruz	sc-552
TIMP3	Cytoplasm	Oncogene	IM43T

Statistical analysis

A comparison of the proportion was performed using Pearson ^{2} test or the Fisher's exact method. Analysis of methylation status vs protein expression was performed using the contingency coefficient.

RESULTS

Among the 65 CRC patients, there were 39 SCRCs, 10 HNPCCs and 16 colorectal carcinoma in Bethesda category. In addition, eight colorectal adenomas, five non-cancerous mucosa tissues and one normal mucosa sample were also recruited for this study.

Twenty four genes among the 31 targets studied in this study were previously analyzed in liver cancer[10] and astrocytoma (unpublished data) in this and other laboratories. They were APC, BRCA1, CDH1, CDH13, DAPK1, hMLH1, p14[ARF], p15[INK4b], p16[INK4a], p27[KIP1], p73, PTEN, RAR-b, RASSF1A, RASSF1C, VHL and WT1. The CDX1, COX2, CXX1, MINT1, MINT31 and TIMP3 genes have also been studied in colon cancer, the methylation pattern of which was found altered in CRC[16] so that the relevant inter-study comparison of the methylation behaviors should be possible. In this list, there were genes with the proven roles in as well as those lacking any obvious association with the carcinogenesis of human tumors. For the later group, hypermethylation of the myogenetic lineage-specific transcription factor: MYOD1, occurs more frequently in aging tissues than its younger counterparts[17]. In the category of cancer associated genes, the tumor suppressor genes made up the major part. There were proteins operating in the RB1/p16[INK4a] pathway: p14[ARF], p15[INK4b] and p16[INK4a], as well as the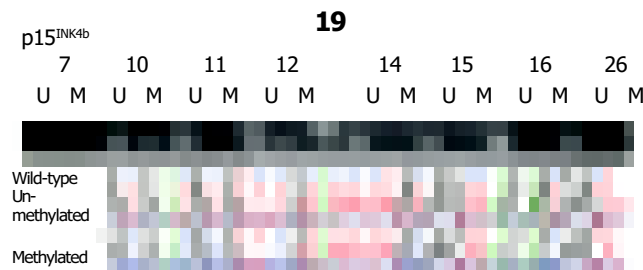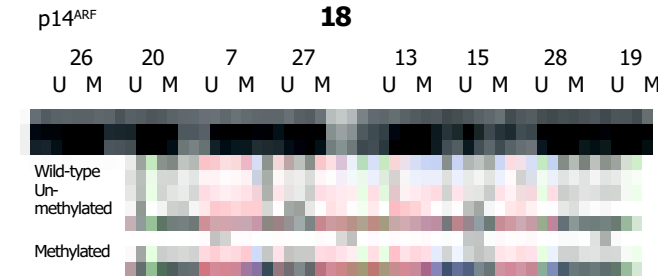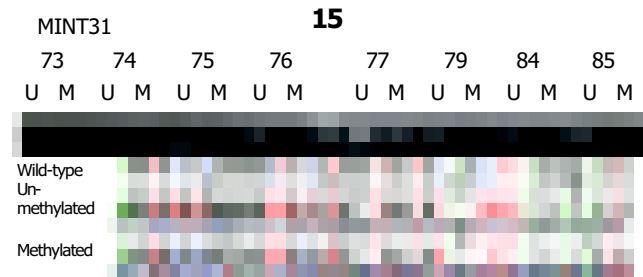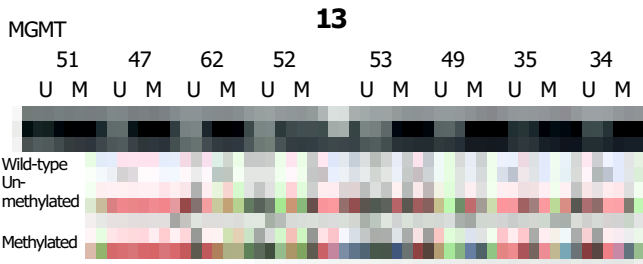 three cyclin-dependent kinase inhibitors: p21[WAF1], p27[KIP1][18] and p57[KIP2][19]. The rest in this subset were the p53 analogue, p73[18,20], the two alternative forms of a tumor suppressors in the Ras mediated signal transduction pathway: RASSF1A and RASSF1C[21], VHL[22], APC[23], PTEN[6], N33 as well as the Wilms tumor 1 gene, WT1[24]. Also included were the genes encoding the cell membrane proteins or nuclear receptors acting actively in intercellular interactions: e.g. melanoma specific antigen A1 (MAGEA1)[25], and cadherins, CDH1[26] and CDH13[26]. One gene implicated in signal transduction was cyclin A1[27]. The gene encoding O-6-methylguanine-DNA methyltransferase, MGMT[28] was also included, which plays a key role in the cellular responses to alkalyting agents and heavy metal stresses. The genes acting in DNA repair process were hMLH1[29] and BRCA1[30] as well as the gene acting in apoptosis, DAPK1[31].

1

APC

1	3	5	9	10	11	12	13
U M	U M	U M	U M	U M	U M	U M	U M

Wild-type
Un-methylated

Methylated

2

BRCA1

1	3	4	5	7	9	10	11
U M	U M	U M	U M	U M	U M	U M	U M

Wild-type
Un-methylated

3

CDH1

1	3	4	5	7	9	10	11
U M	U M	U M	U M	U M	U M	U M	U M

Wild-type
Un-methylated

4

CDH13

29	30	31	33	34	35	38	41
U M	U M	U M	U M	U M	U M	U M	U M

Wild-type
Un-methylated

Methylated

5

CDX1

2	3	4	5	10	11	13	17
U M	U M	U M	U M	U M	U M	U M	U M

Wild-type
Un-methylated

Methylated

6

COX2

2	3	9	13	52	54	55	59
U M	U M	U M	U M	U M	U M	U M	U M

Wild-type
Un-methylated

Methylated

7

CXX1

21	23	24	25	26	27	29	30
U M	U M	U M	U M	U M	U M	U M	U M

Wild-type
Un-methylated

Methylated

8

cyclin A1

29	30	31	33	34	35	37	38
U M	U M	U M	U M	U M	U M	U M	U M

Wild-type
Un-methylated

Methylated

9

DAPK1

67	69	70	71	73	74	75	76
U M	U M	U M	U M	U M	U M	U M	U M

Wild-type
Un-methylated

10

DNMT1

1	2	3	5	7	9	10	11
U M	U M	U M	U M	U M	U M	U M	U M

Wild-type
Un-methylated

11

hMLH1

3	4	7	10	24	25	27	30
U M	U M	U M	U M	U M	U M	U M	U M

Wild-type
Un-methylated

Methylated

12

MAGEA1

35	48	66	84	54	40	41	42
U M	U M	U M	U M	U M	U M	U M	U M

Wild-type
Methylated

13

MGMT

51	47	62	52		53	49	35	34
U M	U M	U M	U M		U M	U M	U M	U M

Wild-type
Un-methylated

Methylated

14

MINT1

9	10	11	12		13	14	15	16
U M	U M	U M	U M		U M	U M	U M	U M

Wild-type
Un-methylated

Methylated

15

MINT31

73	74	75	76		77	79	84	85
U M	U M	U M	U M		U M	U M	U M	U M

Wild-type
Un-methylated

Methylated

16

MYOD1

1	3	4	5		7	9	10	11
U M	U M	U M	U M		U M	U M	U M	U M

Wild-type
Un-methylated

Methylated

17

N33

1	3	4	5		7	9	10	11
U M	U M	U M	U M		U M	U M	U M	U M

Wild-type
Un-methylated

Methylated

18

p14^{ARF}

26	20	7	27		13	15	28	19
U M	U M	U M	U M		U M	U M	U M	U M

Wild-type
Un-methylated

Methylated

19

p15^{INK4b}

7	10	11	12		14	15	16	26
U M	U M	U M	U M		U M	U M	U M	U M

Wild-type
Un-methylated

Methylated

20

p16^{INK4a}

2	3	4	7		9	10	11	12
U M	U M	U M	U M		U M	U M	U M	U M

Wild-type
Un-methylated

Methylated

21

p21^{WAF1}

41	42	43	45		52	53	54	55
U M	U M	U M	U M		U M	U M	U M	U M

Wild-type
Un-methylated

22

p27^{KIP1}

41	42	43	45		52	53	54	55
U M	U M	U M	U M		U M	U M	U M	U M

Wild-type
Un-methylated

23

p73

39	40	41	42		43	45	46	47
U M	U M	U M	U M		U M	U M	U M	U M

Wild-type
Un-methylated

Methylated

24

PTEN

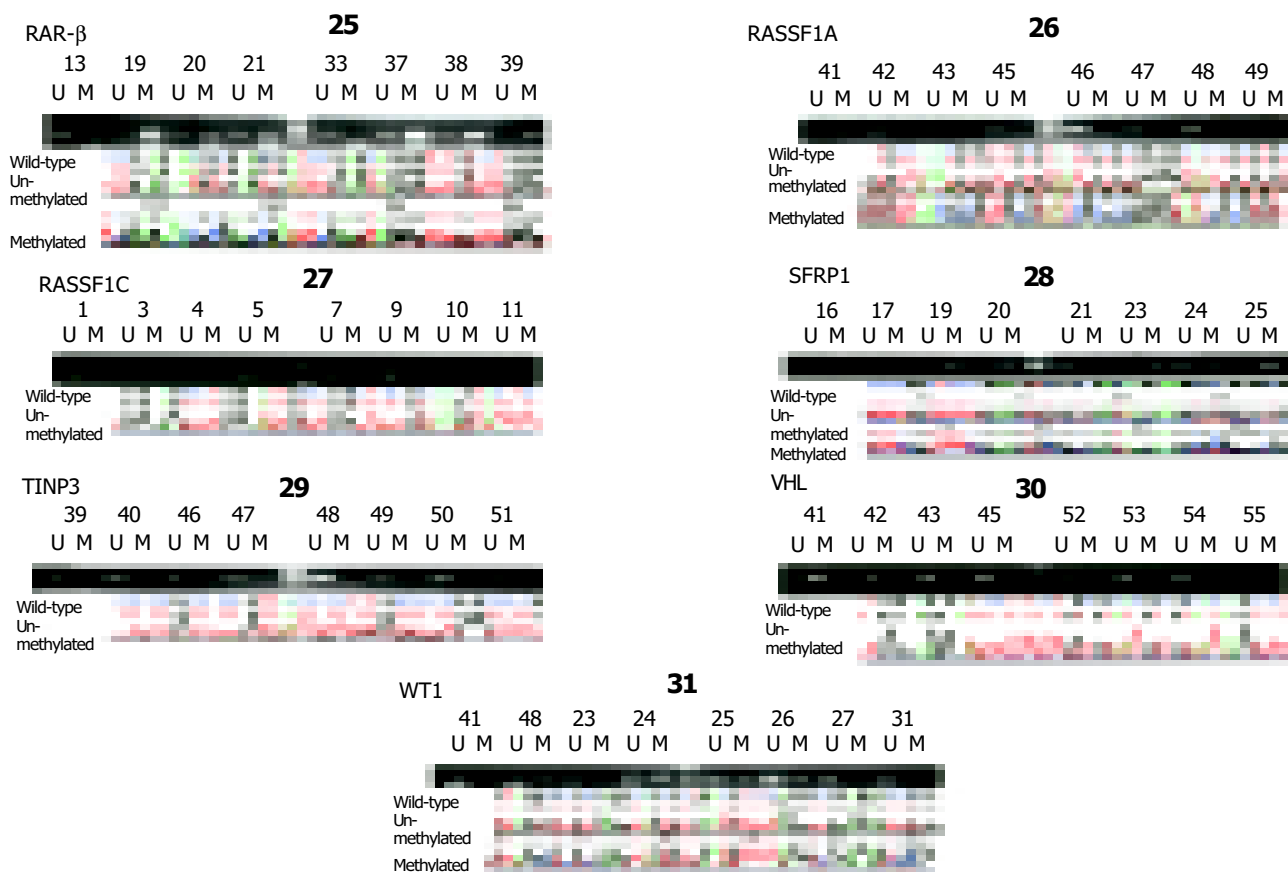| 48	49	50	52		53	54	55	57
U M	U M	U M	U M		U M	U M	U M	U M

Wild-type
Un-methylated

Figure 1 Methylation profiles of the promoter CpG islands of 31 genes in CRC. Both electrophoretic patterns of the representative PCR products of each of 31 targets (indicated respectively, at the top of figures) and the sequencing verification of the one representative PCR product are presented. To indicate the methylation status, the sequenced data are aligned with the wild-type sequence. [1]Size markers, the bands of 250 bp and 100 bp are shown. U, the unmethylated; M, hypermethylated. Panels: 1, *APC*, 2, *BRCA1*, 3, *CDH1*, 4, *CDH13*, 5, *CDX1*, 6, *COX2*, 7,*CXX1*, 8, *cyclin A1*, 9, *DPAK1*, 10, *DNMT1*, 11, *hMLH1*, 12, *MAGEA1*, 13, *MGMT*, 14, *MINT1*, 15, *MINT31*, 16, *MYOD1*, 17, *N33*, 18, *p14^{ARF}*, 19, *p15^{INK4b}*, 20, *p16^{INK4a}* 21, *p21^{WAF1}*, 22, *p27^{KIP1}*, 23, *p73*, 24, *PTEN*, 25, *RAR-b*, 26, *RASSF1A*, 27, *RASSF1C*, 28, *SFRP1*, 29, *TIMP3*, 30, *VHL*, and 31, *WT1*.

Genes displayed no changes in methylation profile in tumor samples

As shown in Figure 1 and summarized in Figure 2, by comparing with the normal mucosa of non-cancerous samples, the following 14 genes did not display any tumor associated changes: *BRCA1* (panel 2), *CDH1* (panel 3), *DAPK1*(panel 9), *DNMT1*(panel 10), *MAGEA1*(panel 12), *N33*(panel 17), *p21^{WAF1}* (panel 21), *p27^{KIP1}* (panel 22), *PTEN* (panel 24), *RAR-* (panel 25), *RASSF1C* (panel 27), *SFRP1* (panel 28), *TIMP3* (panel 29) and *VHL* genes (panel 30). The *MAGEA1* gene was the only target that tends to be demethylated and transcription-activated in tumors, as we previously demonstrated in hepatocellular carcinoma (HCC)[10]. But the hypermethylated state of its promoter CpG islands maintained in all the samples tested. The likely explanations for such no-change-type-observations were the following: no changes in expression of this set of genes occurred in CRC, and the changes in expression indeed occured, but the DNA methylation mediated mechanisms was not involved.

It should be pointed out that our no change conclusion as to both *N33* and *TIMP3* genes was at odds with the previous report where both genes were hypermethylated rather frequently in CRC[32]. Whether it was attributed to the inherent difference between patient groups in different studies remains to be clarified.

Genes displayed changes in methylation profile in tumor samples

To score the changes in each CRC case, the methylated status in the mucosa tissue of the non-cancerous patients was taken as the reference, any deviation from which was scored as positive (increasing methylation extent) or negative (decrease in methylation extent).

Seventeen genes in this list displayed to various extents the tumor associated changes. As shown in Figures 1, 2, the changes of the following genes occurred marginally, and their impact on CRC looked trivial: *APC* (panel 1) (8%, 5/65), *RASSF1A* (3%, 2/65) (panel 26) and *p14^{ARF}* (6%, 4/65) (panel 18). It was surprising to see that the extremely low frequency in hypermethylation of the *RASSF1A* gene in CRC, which was hypermethylated in a wide range of the human tumors, including hepatocellular carcinoma[10,14], astrocytoma (unpublished results) and other tumors[33,34].

The following genes displayed significant alterations in methylation pattern deviated from that of the mucosa of the non-cancer tissues, including those with the moderate levels of changes: *MGMT* (20%, 13/65, panel 13*)*, *hMLH1* (18%, 12/65, panel 11), *p16^{INK4a}*(10%, 10/65, panel 20), *MINT1* (15.4%, 10/65, panel 14), *MINT31* (11%, 7/65, panel 15); and with a great level of changes: *COX2* (72%, 47/65, (panel 6), *cyclin A1* (100%, 65/65, panel 8) and *CDX1* (100%, 65/65, panel 5), *RAR-* (85%, 55/65, panel 25), *MYOD1* (69%, 45/65, panel 16), *p15^{INK4b}* (68%, 44/65, panel 19), *CDH13* (66%, 43/65, panel 4), *CXX1* (58%, 38/65, panel 7), *p73* (63%, 41/65, panel 23) and *WT1* (58%, 38/65, panel 31, Figure 1). In this list, while changes in methylation pattern of the majority of targets, were compatible with the previously reported frequencies in CRC patients in the Western countries[7,11,35,36], there was a noticeable difference. For instance, we have found the *p15^{INK4b}* was significantly methylated in CRC (68%, 44/65), while this gene was not methylated at all in a similar study in USA[35].
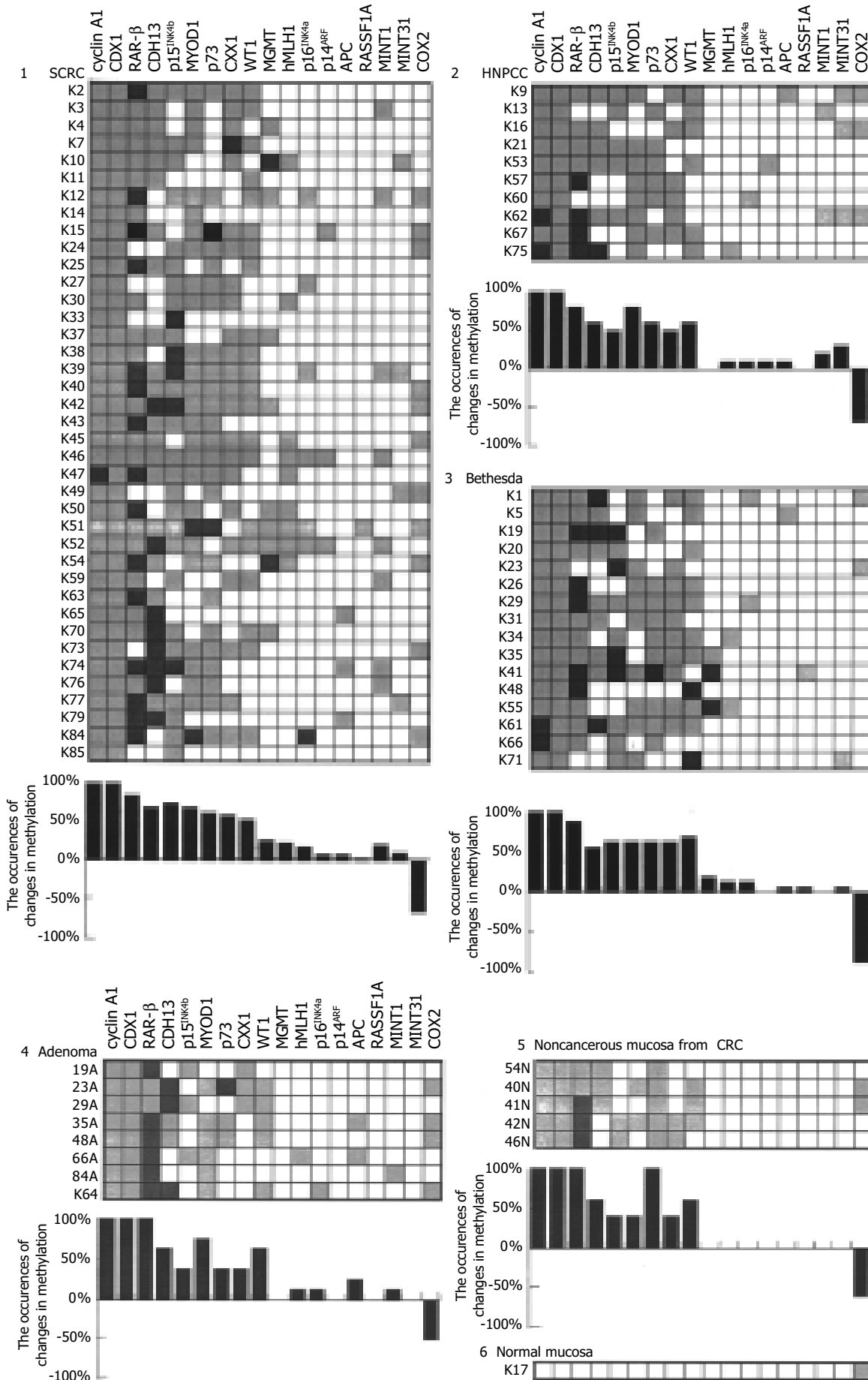
Figure 2 Changes in methylation pattern of genes in CRC and other relevant tissues. The frequency (%) of the hypermethylated targets among the total cases was calculated, and the frequency of the changes in the methylation pattern is presented in the plot as well as in the attached table. Panels: 1, SCRC, 2, HNPCC, 3, Bethesda, 4, Adenoma, 5, Non-cancerous mucosa from CRC patients and 6, the normal mucosa from a non-cancerous patient.

It was also our intention to correlate the methylation pattern with the clinical-pathological features of CRC patients. Subjected to the stringent statistic analysis ($P<5\%$), none of the comparisons displayed any significant correlation. For instance, there was no statistic significance between the methylation changes of any given genes among different subgroups of CRC: SCRC and HNPCC as well as against other cohorts of tissues, such as colorectal adenoma and adjacent mucosa. This was likely to attribute to the small sample size. Although there was a decent number for SCRC ($n = 39$), the sample sizes for other subgroups were rather small: 10 HNPCCs, 16 Bethesda CRCs, eight adenomas, five non-cancerous mucosae and one normal mucosa.

It has been well recognized that the so-called non-cancerous cells pathologically defined may have already suffered some genetic lesions as the corresponding cancerous tissues, the outcome of the earlier events of carcinogenesis. The scenario of the genetic events from the normal mucosa to the full-blown CRC within the context of the well defined clinical and pathological parameters has been well characterized[37]. In this study, we included samples of non-cancerous mucosa from five CRC patients, adenomatous samples from eight patients and tumorous tissues from 65 CRC cases including 39 SCRCs, 10HNPCCs and 16 Bethesda CRCs, which enabled us to determine whether the changes in methylation pattern in CRC were specific to the early or late phases of carcinogenesis. Judged by the no difference in methylation pattern between the non-cancerous mucosa and adenoma and /or CRC (Figures 1, 2) (the same rationale used in our previous work on the hepatocellular carcinoma[10]), we suggested that the high frequent changes in methylation pattern of the genes might be the early rather than the late phase events during carcinogenesis of CRC.

CRC was among the first few types of human cancers subjected to the rather intensive DNA methylation profiling, where changes in methylation in tumor samples were classified as the aging related as well as the cancer specific[17,38,39]. For instance, the *MYOD1* was found also hypermethylated more frequently in the aged healthy epithelia tissues than their younger counterparts[17]. Also from the studies of CRC, the concordant methylation profile of multiple genes was firstly reported, called "CpG island methylation phenotype (CIMP)", short for CpG island methylator phenotype[11]. It implies that the unknown genetic mechanism (s) operating in CRC may contribute to the clustering profile of the DNA methylation of the multiple genes together. Among the 65 CRC cases, the distribution in occurrence of changes in the multiple genes was as following, 1, 3, 5, 7, 12, 11, 11, 7, 4, 1, and 3 cases, for assuming the methylation change of from 2, progressing step by step to 12 genes, respectively (Figure 3), with the 7 gene group as the peak.

In situ expression profile of 10 genes in CRC

Hypermethylation of the promoter CpG islands could reflect

the transcriptional silencing status of the gene. However, exceptions also were reported, suggesting the involvement of other mechanisms in the control of the gene transcription. To verify this notion, we immunostained 10 target proteins *in situ* on the home-made tumor tissue arrays, where the tissue blocks from 58 CRC samples along with several controls of the non-cancerous mucosa from the cancer patients were present. The results were scored from the negative (-), to positive: ranging from + to +++. With the expression level in the non-cancerous mucosa as reference, the relative level of expression of each protein in the context of the methylation state of the promoter CpG islands of the genes was assessed. As shown in Figure 4 and summarized in Table 5, the unmethylated states of the promoter CpG islands of the following genes were indeed positively correlated with the expression levels of the genes. Among the cases of the fully unmethylated targets, expression of each protein $\geqslant$ one + level occurred very frequently: for the *CDH13*, 73% (16/22); *RAR-b*, 100% (8/8); COX2, 80% (35/44); *p73*, 60% (12/20); *hMLH1*, 75% (35/47); *p14^{ARF}*, 100% (51/51); *TIMP3*, 80% (46/58) and *CDH1*, 98% (57/58). The only exception was *MGMT*, only 25% (2/8) of the unmethylated cases expressed this protein, suggesting the mechanisms other than DNA hypermethylation being involved. For the CRC cases with the heterozygous methylated status (u/m), expression of each proteins was: *CDH13*, 50% (14/28); *cyclin A1*, 70% (37/53); *RAR-*, 79% (22/28); *COX2*, 100% (14/14); *p73*, 54% (19/35); *MGMT*, 24% (11/46); *hMLH1*, 36% (4/11) and *p14^{ARF}*, 100% (2/2), respectively. For the CRC cases with the fully methylated targets, the expression profile of the genes was as the following: *CDH13*, 38% (33/58), *cyclin A1*, 80% (4/5), *RAR-*, 85% (17/20) and *MGMT*, 50% (2/4). Except for the *CDH13*, which expressed less frequently in the methylated than the unmethylated containing cases, the expression frequency was not significantly less prevalently in the methylated cases, even higher as far as the *MGMT* was concerned. Therefore, the correlation between the methylation status and level of expression of these 10 genes was not in a good agreement with the conventional notion.
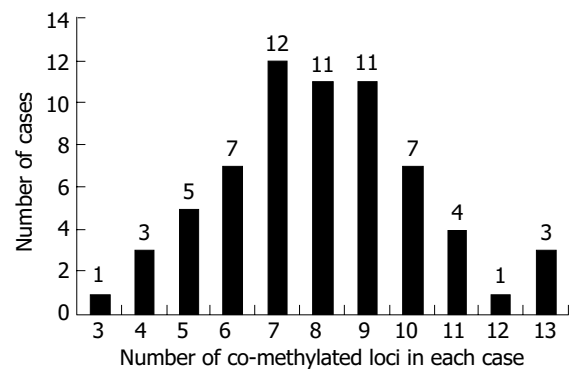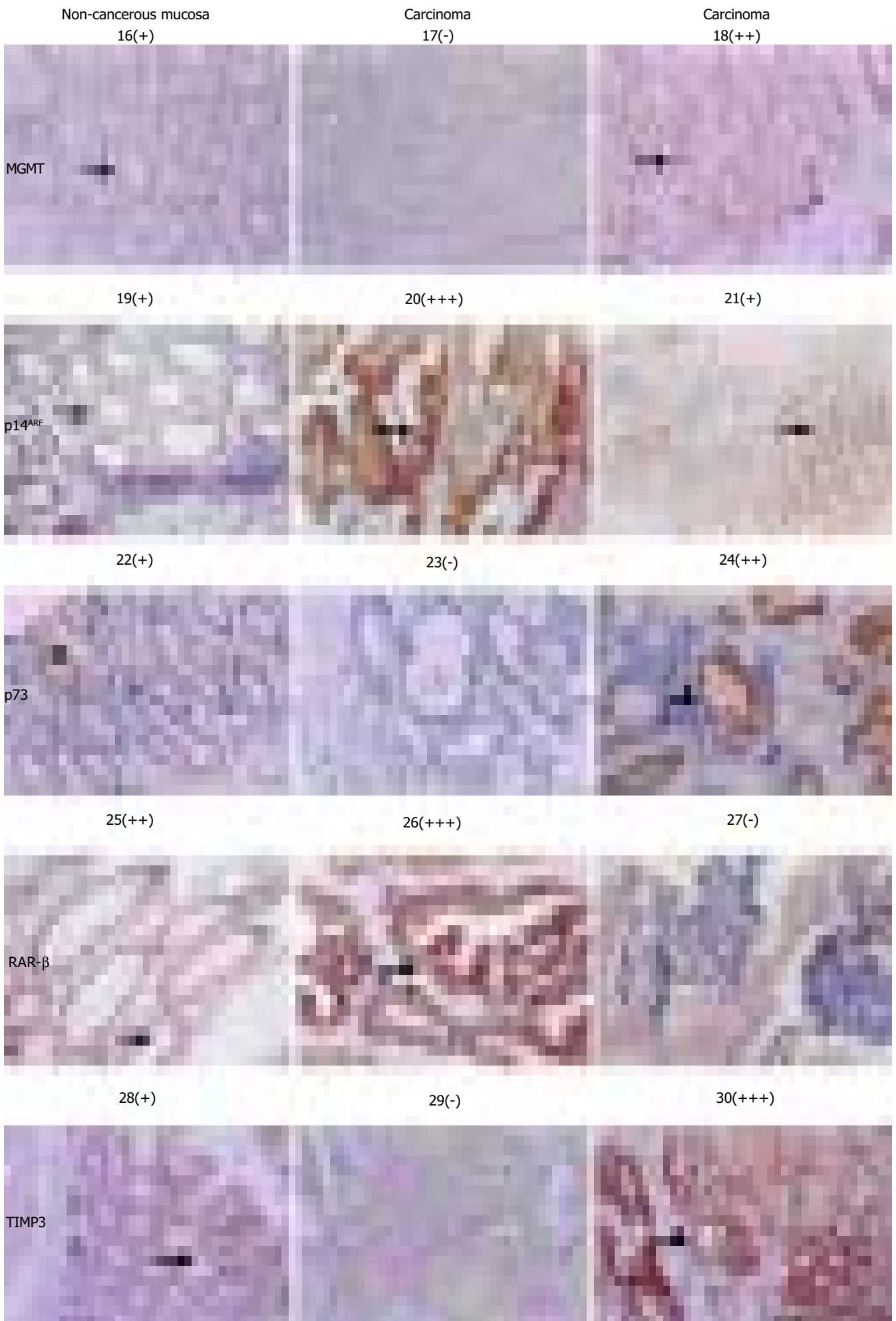

Figure 3 Concordant behavior of the multiple methylated loci in CRC.

Table 5 Expression profile of the genes in the context of DNA methylation status

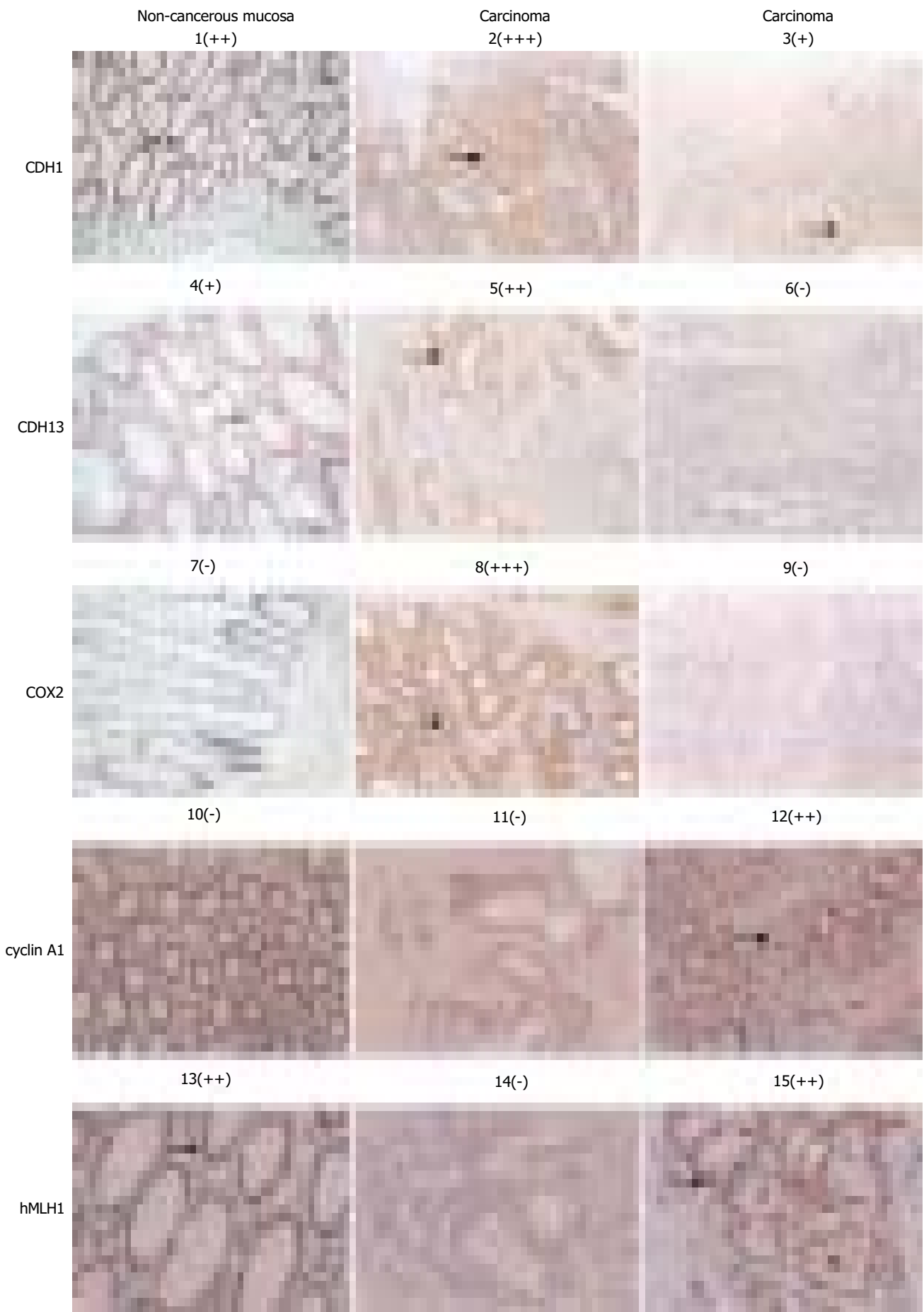| Methylation status	Positive rate % (Positive cases/the total cases)									
	CDH13	Cyclin a1	RAR-	COX2	P73	MGMT	hMLH1	p14ARF	TIMP3	CDH1
U	73 (16/22)	/	100 (8/8)	81 (35/43)	60 (12/20)	24 (11/46)	74 (35/47)	100 (51/51)	80 (46/58)	98 (57/58)
U/M	50 (14/28)	70 (37/53)	79 (22/28)	93 (14/15)	54 (19/35)	25 (2/8)	36 (4/11)	100 (2/2)	/	/
M	38 (3/8)	80 (4/5)	85 (17/20)	/	/	50 (2/4)	/	/	/	/
Total	57 (33/58)	71 (41/58)	84 (47/56)	84 (49/58)	53 (31/58)	26 (15/58)	67 (39/58)	100 (53/53)	79 (46/58)	98 (57/58)
Non-cancerous mucosa	+	/	++	/	+	+	++	+	+	++

Non-cancerous mucosa	Carcinoma	Carcinoma
16(+)	17(-)	18(++)

MGMT

19(+)	20(+++)	21(+)

p14^{ARF}

22(+)	23(-)	24(++)

p73

25(++)	26(+++)	27(-)

RAR-β

28(+)	29(-)	30(+++)

TIMP3

Figure 4 Immunochemical assessment of the expression level of each of 10 target genes. Both the protein targets (left site to) and sample identities (top down) of the immuno-chemical stained picture are indicated. The signs: -, +, ++, and +++ in bracket are used to provide the quantitative reference in pictures below. The areas pointed by the arrows are the regions showing the immunostaining data quantified.

DISCUSSION

In this study, 31 targets were selected to be studied, 65 cases of CRC, 8 cases of adenoma, five cases of non-cancerous mucosa from the cancer patients and one case mucosa from the non-cancerous patient. As far as the number of genes was concerned, this study is rather extensive, and it is the first of such a kind with CRC patients in China, and in Shanghai in particular.

Changes in methylation pattern of the promoter CpG islands are very common in CRC

By taking the methylation profile of the mucosa from the non-cancerous patient as the reference, CRC associated changes in methylation pattern were a very extensive event, 17 among of 31 targets assumed the altered patterns, the following 14 changed in more than 20% of CRC cases as a whole: $MGMT$ (20%, 13/65), $hMLH1$ (18%, 12/65), $p16^{INK4a}$ (10%, 10/65), $MINT1$ (15.4%, 10/65), $MINT31$ (11%, 7/65), $COX2$ (72%, 47/65), $cyclin A1$ (100%, 65/65), $CDX1$ (100%, 65/65), RAR- (85%, 55/65), $MYOD1$ (69%, 45/65), $p15^{INK4b}$ (68%, 44/65), $CDH13$ (66%, 43/65), $CXX1$ (58%, 38/65), $p73$ (63%, 41/65) and $WT1$ (58%, 38/65) genes. Furthermore, there was only one case assuming the methylation changes with three genes (Figure 3), no less than 76.3 % of cases assumed changes in methylation more than seven targets (49/65), 86% more than six targets (56/65), 93.8% more than five targets (61/65). This observation indeed supports the concept, "CpG island methylator phenotype", for that changes in methylation pattern in CRC indeed tend to be clustered. As a similar survey with the astrocytoma showed that less 26.4% patients assumed no more than two gene (among 31 targets being surveyed), and the occurrence of changes in methylation pattern for at least five, six and seven targets was 41.51%, 20.75 and 9.43% in a cohort of 56 astrocytoma patients (unpublished data). The high incidence of changes in CRC may be partly attributed to the fact that colorectal epithelial cells were much amenable to the environmental challenges.

Except for the genes that have been proved in the category of tumor associated genes, such as $p16^{INK4a}$, $p15^{CDKN4b}$, $p14^{ARF}$, $p27^{KIP1}$, $WT1$, VHL, RB, $p73$, APC, $N33$, $PTEN$ and $BRCA1$, the $MAGEA1$ and RAR- genes have not been studied in CRC. While the $MAGEA1$ gene displayed the homozygously methylated state in all samples tested, the RAR- gene indeed changes its methylated state from the unmethylated in the normal mucosa toward the hypermethylated state at a significantly higher frequency in CRC, over 85% (55/65). The retinoic acid receptor (RAR) gene is a putative tumor suppressor gene on chromosome 3p24, where a high incidence of loss of heterozygosity (LOH) is detected in many types of tumors. Retinoic acid suppresses cancer cell growth through binding to RARs, especially RAR-, indicating a critical role in mediating anticancer effects. Selective loss or down-regulation of the RAR mRNA and protein has been reported in prostate cancers. The DNA methylation mediated silencing of this gene has been suggested as an etiological factor for carcinogenesis of prostate cancer[40]. This result indicates that the RAR- hypermethylation may be the mechanism of expression silencing in these tumor cells[41]. However, its etiological significance in carcinogenesis of CRC remains to be clarified.

The following genes: $CXX1$, $CDX1$, $SFRP1$, $MINT1$ and $MINT31$ were initially identified as the CRC associated hypomethylated genes by an approach at the genome level[16]. Except for the $SERP1$ gene that was not methylated with all the samples being tested, the rest targets were indeed hypermethylated to varying extents in CRC (Figures 1, 2). Tumor suppressor gene $RASSF1A$ was inactivated predominantly by promoter methylation and rarely by somatic mutations. wagner et al.[34] investigated RASSF1A promoter methylation in colorectal cancer and detected RASSF1A methylation in 80% (4/5) colorectal cancer cell lines and 45% (13/29) primary colorectal cancers. But we found that the frequency of $RASSF1A$ methylation was extremely low in CRC although we previously found this gene was hypermethylated (100%) in HCC[14].

Does the methylation profile of the promoter CpG islands of the genes correlate with the expression of the genes?

Although it has been generally accepted that the hypermethylated status of the promoter CpG islands represents the long-term transcription silencing state of the genes, its credential has not been thoroughly checked in clinical samples, due to various limiting factors, including availability and inherent diversity of the cell types in the actual samples. In this study, we immuno-stained the genes with antibodies to assess the expression of 10 genes in a home-made tissue array. For both homozygously and heterozygously unmethylated samples, nine of 10 targets (except for $MGMT$) expressed at a detectable level of more than 60% cases. It was generally fit with the expectation. For the fully methylated CRC, only four targets were involved, among which the profile of the $CDH13$ gene met the expected profile. The expression frequency (38%, 3/8) in methylated samples was significantly lower than heterozygously methylated (50%, 14/28), and homozygously unmethylated (73%, 16/22). The profiles of the other three targets did not comply with the role at all. Both $cyclin A1$ and RAR- genes expressed equally well as the CRC samples of m/u type. Furthermore, it was paradoxically found that the $MGMT$ gene expressed more prevalently in the methylated CRC cases (50%, 2/4) than its counterparts (u/m: 25%, 2/8 and u: 25%, 11/46). As we have previously shown in astrocytoma cell lines, the methylated status of the promoter CpG islands inversely correlated with the expression of the $MGMT$ gene (unpublished data), this rather unexpected observation should be looked into further. In conclusion, despite the general roles that have been followed as to the inverse correlation between the methylation state of the promoter CpG islands and expression, the exception is also distinguishingly noticeable. Therefore, it should be very cautious in correlating the methylated status with the expression profile of the genes.

Demethylation of the COX2 gene correlates with the increased frequency in its expression

Cyclo-oxygenase 2 (COX2), an inducible isoform of prostaglandin H synthase, which mediates prostaglandin synthesis during inflammation, and is selectively over-expressed in colon tumors, is thought to play an important role in colorectal carcinogenesis[42]. Its expression in the normal epithelial cells is low, but can be induced by a variety of stressing agents, including tumor promoters and inflammatory cytokines. In the list of the genes displaying changes in methylation, it was noticed that the $COX2$ gene was the only target that displayed CRC associated demethylation, i.e., from the heterozygous methylated state to fully demethylated in 47 among 65 cases being studies (72%) (Figures 1, 2). The $COX2$ gene did not express in the control non-cancerous tissues, indicating that it seems less likely to have the physiological role in normal colon epithelial cells. Although there was no increase in the frequency of expression in CRC from the heterozygously to the fully unmethylated CRC cases (Figure 2), the fact that over 82% CRC cases expressed this gene was indeed supportive.

Tumor type specificity of the methylation profile

It has been well established that the methylation profile as an inheritable epigenetic signature in any given cells reflects both history of cell differentiation and the chronicle of aging, in addition to those closely associated with the biological profiles

of tumors[6,43,44]. It is desirable to identify the changes, specific to one type of tumor from the others, otherwise our efforts to use the DNA methylation pattern, as the biomarkers for tumor diagnosis would be severely constrained. Therefore, we compared the methylation patterns in HCC[10,14] with those in CRC. As shown in Figure 5, there were methylation changes common to both HCC and CRC i.e., the *p73* gene: 73% in HCC *vs* 62.5% in CRC and the *MYOD1* gene: 57.8% in HCC *vs* 69.23% in CRC. The following changes were more prevalent in CRC than in HCC: the *cyclin A1* gene: 100% in CRC *vs* 53.85% in HCC; the *WT1* gene: 58.46% in CRC *vs* 30.77% in HCC; and the *CDH13* gene: 64.62% in CRC *vs* 23.08% in HCC. The following two genes were more frequently hypermethylated in HCC than in CRC: the *RASSF1A* gene: 100% in HCC *vs* 2.08% in CRC; and the *p16^{INK4a}* gene: 53.85% in HCC *vs* 15.38% in CRC. Although little is known about the mechanisms underlying the tumor specific methylation profiles of CRC *vs* those of HCC, the distinct profiles of CRC *vs* HCC should have the profound implication in the future diagnostic tools to differentiate these two common human cancers.
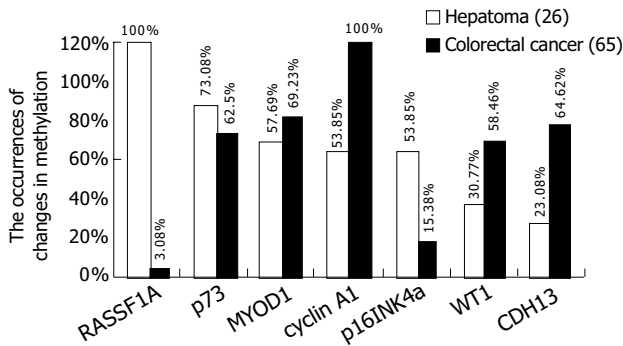
Figure 5 Tumor specific methylation pattern: CRC verse HCC.

In conclusion, the altered promoter CpG island methylation patterns of 17 genes are the common and distinguished hallmarks specific to the early phase of colon carcinogenesis, although no significant correlation has been detected between the changes in the methylation patterns with any given clinical-pathological features. The correlation between the methylated state of the promoter CpG islands and the expression of the corresponding genes has not been proved as close as previously suggested. Our observations from this rather extensive methylation-profiling maneuver would provide new insights into the role of the DNA methylation mediated control in the colon carcinogenesis as well as the clues for the development of robust diagnostic and prognostic tools for colorectal cancer.

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